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Induction of TCR Gene Rearrangements in Uncommitted Stem Cells by a Subset of IL-7 Producing, MHC Class II–Expressing Thymic Stromal Cells

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Summary

The embryonic thymic microenvironment provides the necessary elements for T cell lineage commitment, but the precise role of individual stromal cell components remains to be determined. Here we address the question of which stromal cell types are required for initiation of V–DJ rearrangements of the TCR-β and TCR-δ locus in CD117$^+$CD45$^+$ uncommitted fetal liver progenitors. We show that fetal thymic stroma alone is necessary and sufficient for induction of TCR-β and TCR-δ rearrangements. Furthermore, the ability to induce this T cell commitment step is confined to a subset of MHC class II–positive epithelial cells. Thymic stroma derived from mice with a targeted deletion in the IL-7 gene, however, lacks this ability. These findings set the stage for a further definition of the nature of the thymic stromal cell support in the regulation of T cell commitment.

Introduction

T cell development follows a highly regulated program that begins during embryonic life in the thymus. In the mouse, fetal liver–derived stem cells enter the rudimentary thymic stroma from day 12 of gestation and progress through a series of distinct maturational stages, eventually to become mature T cells and leave the thymus (reviewed by Shortman and Wu, 1996; Zúniga-Pflücker and Lenardo, 1996). This process is driven by interactions between developing thymocytes and thymic stromal cells (reviewed by Anderson et al., 1996), which provide cytokines, extracellular matrix products, and cell surface ligands. At the same time, differentiation of the thymic stroma is dependent on distinct stages of developing thymocytes (Ritter and Boyd, 1993; Shores et al., 1994; Van Ewijk et al., 1994; Boehm et al., 1995; Holländer et al., 1995; Anderson et al., 1996). The complexity of the thymic microenvironment has so far precluded a definition of the precise roles of individual stromal cell components in the various developmental transitions that stem cells undergo during intrathymic differentiation and lineage determination. Here we report on the nature of the stromal cells that support commitment of fetal liver stem cells to the T cell lineage through induction of V–DJ rearrangements in the TCR-β and TCR-δ locus.

The intrathymic precursor pool includes multipotent stem cells (Shortman and Wu, 1996; Zúniga-Pflücker and Lenardo, 1996) that can also give rise to non-T cells such as dendritic cells, natural killer cells, and B cells. This earliest intrathymic precursor has been defined as a CD117 (c-kit)$^{++}$CD44^{++}CD25$^-$ population in which the TCR-β, γ, and δ loci are still in germline configuration, but products of the recombinase-activating genes (RAG-1 and RAG-2) are already expressed (Godfrey et al., 1994; Wilson et al., 1994). Loss of CD117 and CD44 and gain of CD25 correlates with initiation of rearrangements at the TCR-β, γ, and δ loci (Godfrey et al., 1994; Wilson et al., 1994; Shortman and Wu, 1996; Zúniga-Pflücker and Lenardo, 1996) and loss of multipotency. The subsequently arising CD117$^-$CD44$^-$CD25$^+$ population expresses full-length rearrangements of the TCR-β, γ, and δ loci (Godfrey et al., 1994; Wilson et al., 1994; Moore and Zlotnik, 1995; Zúniga-Pflücker et al., 1995; Shortman and Wu, 1996; Zúniga-Pflücker and Lenardo, 1996), is irreversibly committed to T cell lineage development, and can give rise to both αβ and γδ T cells. Production of a TCR-β chain protein marks the beginning of a series of events including proliferation, induction of CD4 and CD8 expression, loss of CD25 expression, shutdown of TCR-β chain rearrangements, and initiation of TCR-α chain rearrangements. TCR-β does not act alone, but requires participation of the pre-Tα glycoprotein (Saint-Ruf et al., 1994; Fehling et al., 1995), the CD3 components (Malissen et al., 1995), and at least one nonreceptor protein-tyrosine kinase, p56^{lck} (Anderson et al., 1994b; Levelt and Eichmann, 1995). In the absence of pre-Tα (Fehling et al., 1995) or TCR-β (Mombaerts et al., 1992a), development of αβ T cells is blocked, while γδ T cell development still occurs (Mombaerts et al., 1992a; Kisielow and Von Boehmer, 1995; Fehling et al., 1995). TCR-β-mediated selection thus represents a permissive step for further T cell differentiation, making an analysis of the requirements for induction of TCR-β rearrangements a valuable hallmark for an investigation of the mechanisms regulating T cell commitment. In addition, we included an analysis of TCR-δ gene rearrangements in this study, given the finding that these precede TCR-β rearrangements.

The precise nature of the signals driving induction of TCR gene rearrangements in uncommitted precursors is unknown. The nonlymphoid part of the thymus is mainly composed of a fine epithelial network, originating from the third pharyngeal cleft and pouch (Bockman and Kirby, 1984). Based on immunohistochemistry studies (Boyd et al., 1993; Anderson et al., 1996), many different types of epithelial cells can be distinguished, including cortical and medullary epithelial cells, epithelial cells that line the mesenchymal capsule of the thymus, and epithelial cells at the cortical–medullary junction. In addition, the thymus includes non-T cells of hematopoietic origin, such as macrophages and dendritic cells (Boyd et al., 1993; Surh and Sprent, 1994). Thymus stroma is thus complex in composition, and the regulation of T

lineage commitment, in so far as signals delivered by thymic stromal cells are concerned, is unclear.

Several mutant mouse strains have been described that underline the distinct role of specific subpopulations of nonlymphoid thymic cells. Mice in which the RelB gene has been mutated by homologous recombination exhibit deficiencies in their medullary epithelial and dendritic cells (Burkly et al., 1995; Weih et al., 1995). While these mice are defective in their antigen presentation capacity, TCR rearrangements and subsequent T cell development proceed undisturbed (Weih et al., 1995). Dendritic cells and medullary epithelial cells thus do not appear to exert an effect on thymic T cell development. The T cell–deficient nude mice carries a mutation in the winged-helix (*whn*) gene (Nehls et al., 1994, 1996), leading to a lack of development of the cortical and medullary epithelial compartment of the thymus. That formation of both these areas is in turn affected by thymocytes has been revealed in various other mutant mouse strains. The thymic cortex is severely disturbed in mice transgenic for the human CD3ε gene (Holländer et al., 1995), which exhibit a block at the $CD44^+CD25^-$ precursor stage. This cortical epithelial defect can be restored in fetal mice (but not in adult mice) by transplantation of normal hematopoietic stem cells (Holländer et al., 1995). Interestingly, cortical epithelial cells have been implicated in the transition of the $CD44^+$ $CD25^-$ to the $CD44^-CD25^+$ (i.e., committed) stage of T cell development (Anderson et al., 1993, 1996). RAG-$1^{-/-}$ or RAG-$2^{-/-}$ mice and SCID mice, which exhibit a block at the $CD44^-CD25^+$ stage, exhibit a normal cortical thymic area (Shores et al., 1994; Van Ewijk et al., 1994), but fail to develop a medullary compartment; this defect can be restored by transfer of mature T cells (Shores et al., 1994). Finally, the blockage at the $CD4^+CD8^+$ stage of development observed in TCR-α-deficient mice (Palmer et al., 1993) is associated with the absence of medullary areas.

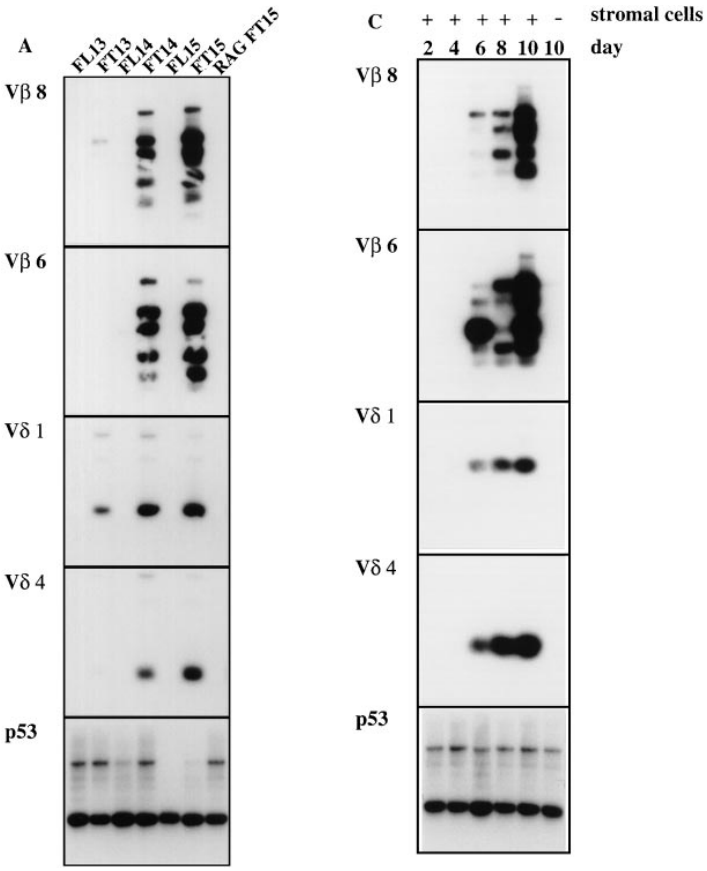
The central question addressed in the present study is the nature of the stromal cell requirements supporting the initiation of TCR-β and δ chain rearrangements in uncommitted precursors. We modified the reaggregate thymic culture model, which has been applied to study development of thymocytes (Jenkinson et al., 1992), by mixing suspensions of day 14 fetal liver stem cells that still have TCR-β and TCR-δ in germline configuration with different thymic stromal cell preparations to reconstruct a thymus lobe. Stromal cell preparations were prepared from thymi of RAG-$1^{-/-}$ mice so as to preclude any contribution of TCR-β or δ gene rearrangements from thymocytes trapped in the stromal cell preparation. Rearrangements of the TCR loci are analyzed over time by polymerase chain reaction (PCR) and are not detectable in fetal liver cells cultured without stroma. In contrast, when cultured with fetal thymic stroma, induction of TCR-β and δ rearrangements occurred in total fetal liver cells and in purified $CD117^+CD45^+$ fetal liver progenitors. The ability to support this T cell commitment step was confined to the class II–positive epithelial population. Epithelial cells from mice with a targeted deletion in the interleukin-7 (IL-7) gene, on the other hand, are unable to induce TCR rearrangements. This approach provides the basis from which the nature of the thymic stromal cell support in T cell lineage commitment can be defined.

Results

Thymic Stromal Cells Are Required and Sufficient for Induction of TCR-β and TCR-δ Rearrangements in Uncommitted Precursors

To investigate whether mouse fetal liver precursors could be induced to differentiate into committed T cell precursors by the thymic stromal cell microenvironment, we employed the reaggregate culture model (Jenkinson et al., 1992) for coculture of fetal liver cells from normal mice and stromal cells derived from fetal RAG-$1^{-/-}$ mice. As a readout for T cell commitment, we assayed V–DJβ and V–DJδ rearrangement by PCR. Figure 1A depicts V–DJ rearrangements of the TCR-β and TCR-δ locus in total fetal liver and fetal thymus from mouse embryos at days 13, 14, and 15 of gestation. Rearrangement of Vδ1 to Jδ1 was first detected in the thymus of a day 13 embryo, consistent with earlier reports (reviewed by Haas and Tonegawa, 1992), while rearrangement of Vβ8 to Jβ2.1 and Vδ4 to Jδ1 rearrangements were only barely detectable. Complete V–DJ rearrangements of the Vβ8 and Vβ6 fragments to the Jβ2 fragments were first present in the thymus at day 14 of gestation. In contrast, no V–DJβ nor V–DJδ rearrangemant could be detected in the fetal liver at any one time. Fetal liver cells thus represent an appropriate source of uncommitted stem cells to be used for an investigation of the regulation of induction of β and δ chain rearrangements. As expected, no rearrangements were observed in a fetal thymus derived from RAG-1-deficient mice (Figure 1A); stromal cell preparations from RAG-$1^{-/-}$ mice will thus not contribute to any rearrangements induced by culture.

Figure 1B illustrates the different components of the culture model used in the present study. Stromal cells were derived from fetal thymi of day 15 RAG-1-deficient mice, after depletion of cells of hematopoietic origin through a combination of deoxyguanosine (dGuo) treatment (Jenkinson et al., 1982) and removal of CD45-positive cells by immunomagnetic selection. A fetal liver cell suspension of C57BL/6 mice at day 13–14 of gestation was mixed with the stromal cells in a 1:1 ratio in reaggregate cultures. After 4 days, Vδ1–Jδ1 rearrangements can already be visualized in some exposures, consistent with the fetal thymus data (Figure 1A), but, by and large, all Vδ and Vβ rearrangements are detectable in reaggregate cultures after 6 days (Figure 1C). In contrast, fetal liver cells cultured alone (Figure 1C) did not give rise to either one of these TCR rearrangements, even though intact reaggregates formed in this case. Also, bone marrow stroma (data not shown) or any one of a large number of long-term thymic stromal cell lines previously described (Izon et al., 1994; Rinke de Wit et al., 1996; data not shown) were unable to induce V–DJβ or V–DJδ rearrangements, even when supplemented with physiological survival factors for T cell progenitors, such as IL-7 and stem cell factor (Peschon et al., 1994; Moore and Zlotnik, 1995; Rodewald et al., 1995; Von Freeden-Jeffry et al., 1995). Thymic stroma is thus necessary and



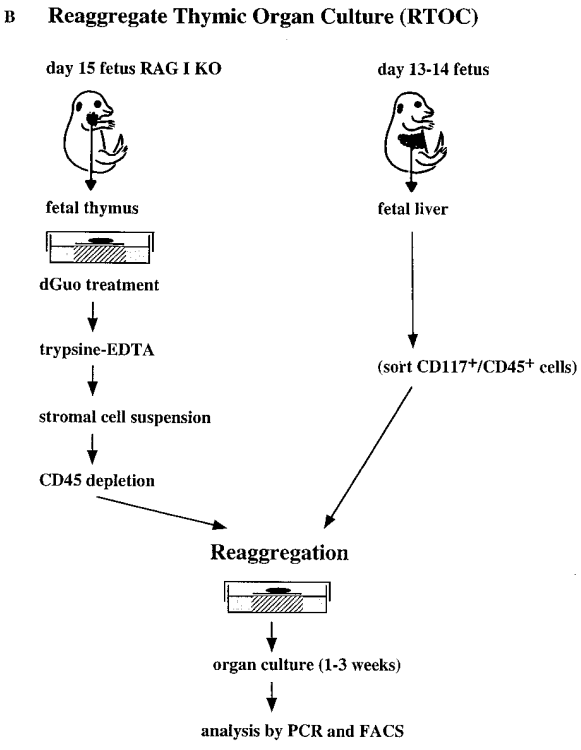


Figure 1. Fetal Thymic Stromal Cells of RAG-1-Deficient Mice Can Induce TCR-β and TCR-δ Rearrangement in Fetal Liver Progenitor Cells in a Reaggregate Thymic Organ Culture

(A) TCR-δ rearrangement is first detected in fetal thymus at day 13, whereas TCR-β starts at day 14. Rearrangement of the TCR-β and δ locus is analyzed in fetal liver and fetal thymus at days 13, 14, and 15 of gestation, using Vβ8- and Vβ6-specific primers in combination with a Jβ2.5 primer. These primers amplify Vβ8/6–Jβ2.1 (850 bp), Vβ8/6–Jβ2.2 (650 bp), Vβ8/6–Jβ2.3 (380 bp), Vβ8/6–Jβ2.4 (240 bp), and Vβ8/6–Jβ2.5 (150 bp) fragments.In addition, Vδ1- and Vδ4-specific primers are used in combination with Jδ1 to amplify 290 and 170 bp rearranged TCR-δ fragments, respectively. PCR products are probed by Southern blot hybridization with a Jβ2-specific or Jδ1-specific probe. Day 15 fetal thymus of RAG-1-deficient mice does not reveal TCR-β or δ rearrangement. Genomic p53 is amplified (640 bp) and hybridized with p53 cDNA probe to demonstrate that proper template was present in each sample.

(B) Schematic representation of a reaggregate thymic organ culture (RTOC).

(C) Rearrangement of TCR-β and δ is observed after 6 days in culture. Reaggregation of fetal liver cells without stroma does not lead to induction of TCR-β or δ rearrangement.

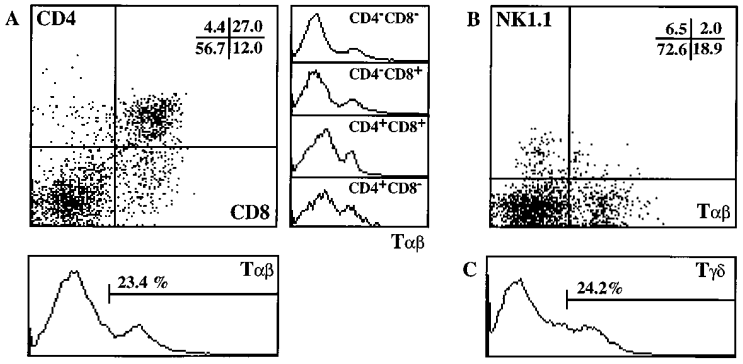


Figure 2. Fetal Thymic Stromal Cells Induce Diverse T Cell Development

Flow cytometry of a RTOC of fetal liver cells and CD45⁻ thymic stromal cells after 13 days in culture. Percentages of subpopulations were calculated using Cell Quest. Shown are CD4-PE, CD8-bio plus SA-Tri, and Tαβ-FITC (A); NK1.1-PE versus Tαβ-FITC (B); and Tγδ-FITC (C).

sufficient for induction of TCR-β as well as TCR-δ chain rearrangements in uncommitted fetal liver stem cells. Importantly, rearrangements using both Vβ6 and Vβ8 fragments could be found (Figure 1C), illustrating that this model system does not selectively favor generation of NK1.1$^+$ T cells, which preferentially express Vβ2, 7, and 8 (Vicari and Zlotnik, 1996).

Fetal Thymic Stroma from RAG-1-Deficient Mice Efficiently Supports Diverse T Cell Development from Fetal Liver Precursors

We next investigated whether the reaggregate culture model also allowed a broader αβ T cell repertoire to develop from fetal liver precursors. The PCR on genomic DNA used above only tested for rearrangements of Vβ8 or Vβ6 segments. These rearrangements represent only part of a normal T cell repertoire and include the subpopulation of T cells that are NK1.1$^+$ and preferentially use Vβ8.2 (Vicari and Zlotnik, 1996). Flow cytometry analysis of CD4 plus CD8 versus TCR-αβ and NK1.1 expression was performed to analyze whether, once TCR-β chain rearrangements had been initiated, further progression of differentiation in fetal liver precursors could be induced in this setting. Figure 2A depicts CD4 plus CD8 expression versus TCR-αβ expression of reaggregate cultures after 13 days. Indeed, both CD4$^+$CD8$^+$ and single positive CD4 and CD8 cells were detected, demonstrating that all subpopulations of thymocytes could be generated through the CD4$^+$CD8$^+$ pathway of T cell development in this setting. Figure 2B illustrates that about 10% of the cells generated express the cell surface antigen NK1.1, but the majority of these cells do not express TCR-αβ. In addition, we analyzed the population of cells generated for the presence of other Vβ family members. The percentages of CD4 or CD8 cells expressing different Vβs was low, but Vβ5, 6, 11, 13, and 14 could clearly be detected (data not shown). Typically, cultures also contained ~30% TCR-γδ cells (Figure 2C). Together, these findings document that fetal thymic stroma contains all components necessary for induction of a diverse T cell repertoire in fetal liver stem cells.

Class II–Positive Thymic Epithelial Cells Are Required and Sufficient for Induction of TCR-β and TCR-δ Rearrangement in Fetal Liver Progenitor Cells

The reaggregate organ culture system is attractive to the extent that it will allow the dissection of the stromal cell types involved in each distinct stage of T cell development. The above findings already allow one conclusion about the requirement for induction of β and δ chain rearrangement: CD45-positive nonlymphoid thymic cells appear to play no role in the induction of this early stage of T cell development, since we routinely removed all such cells of hematopoietic origin by a combination of dGuo treatment and CD45 depletion. We next sorted thymic stromal cells on the basis of class II expression to determine whether CD45-negative stromal cells could be further subdivided with respect to ability to induce TCR-β and TCR-δ chain rearrangements. Staining for major histocompatibility complex (MHC) class II showed a typical biphasic pattern, with about 75% of the cells expressing high levels of MHC class II (Figure 3A). From immunohistological analysis of thymic tissue sections, it appears that all nonhematopoietic class II–positive cells represent epithelial cells, while the class II–negative cells also contain mesenchymal cell populations (Anderson et al., 1996). Figure 3B depicts that omission of class II–positive stromal cells from the stromal population used in reaggregation with fetal liver precursors completely abolished the capacity for induction of V–DJβ and V–DJδ rearrangements. Most importantly, purified class II–positive epithelial cells obtained by cell sorting were sufficient to induce both TCR-β and TCR-δ rearrangement in fetal liver progenitor cells (Figure 3B).

Since MHC class II–positive cells are likely to represent a heterogeneous population (Anderson et al., 1996), we next determined whether they could be further dissected by monoclonal antibodies (MAbs) previously used to discriminate subsets of epithelial cells. The CDR1 (Rouse et al., 1988) and G8.8 (Farr et al., 1991) MAbs distinguish cell surface molecules on cortical and medullary epithelial cells, respectively, in immunohistochemistry studies. However, a flow cytometry analysis in which CD45-negative thymic stromal cells were stained for MHC class II expression versus staining with MAbs G8.8a and CDR1 showed that both these MAbs recognize all MHC class II-bright cells as well as the MHC class II-dull cells (data not shown). These data are consistent with our earlier observation (Izon et al., 1994) that assignments of stromal markers based on tissue section staining do not hold up when flow cytometry is applied. We were also unable to distinguish subsets of class II–positive epithelial cells with NLDC145- (Kraal et al., 1986) and CD40-specific (Heath et al., 1994) reagents: both stained all class II–positive stromal cells

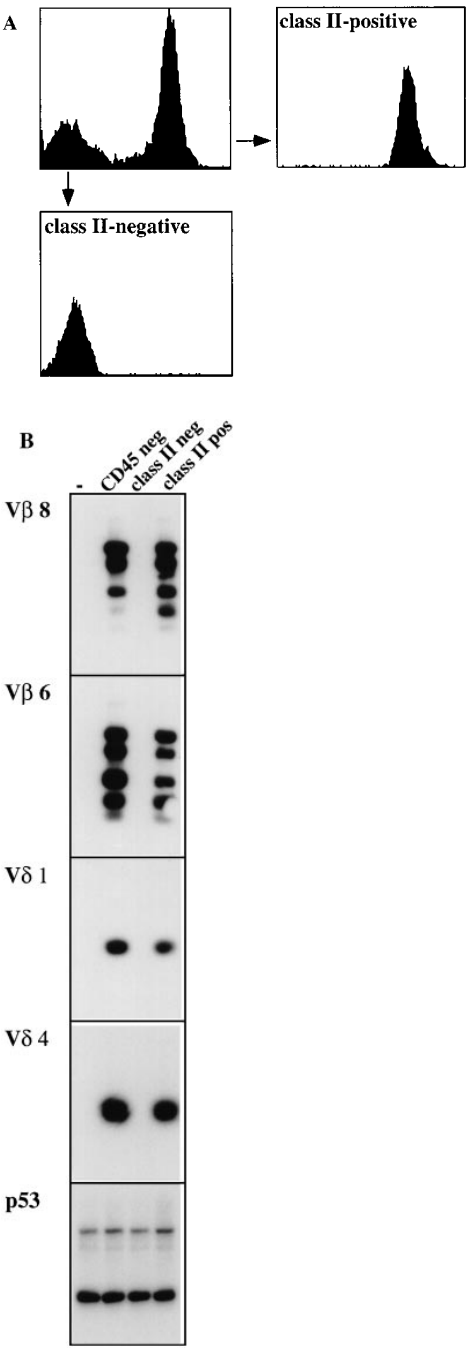


Figure 3. Class II–Positive Thymic Epithelial Cells Are Required for Induction of TCR-β and TCR-δ Rearrangement

PCR analysis of RTOC of sorted subpopulation of stromal cells after 10 days in culture. Fetal thymic stromal cells are depleted for CD45-positive cells and stained with M5/114 supernatant and sheep anti–rat immunoglobulin-FITC. Class II–positive and class II–negative cell populations are sorted to >99% purity. One representative experiment out of six independent ones is shown.

(A) Histogram plot of stained cells and sorted subpopulations.

(B) PCR analysis performed after 10 days for Vβ8, Vβ6, Vδ1, Vδ4, and p53.

(data not shown; see also Izon et al., 1994). At present, no MAbs are thus available that allow us to discriminate subpopulations of thymic epithelial cells by flow cytometry, nor can we isolate distinct subpopulations based

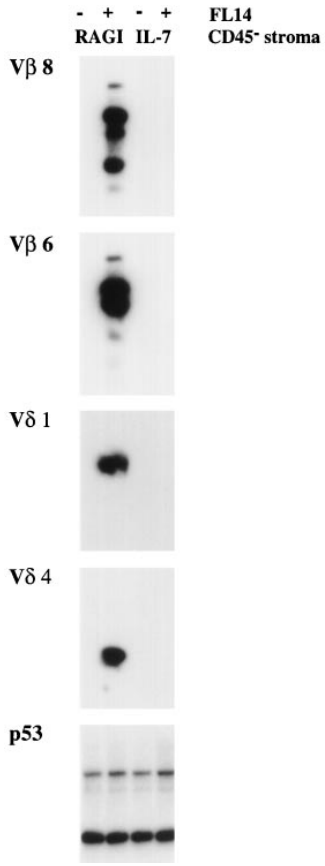


Figure 4. Only IL-7-Producing Thymic Stromal Cells Have the Ability to Induce TCR Gene Rearrangements

PCR analysis of RTOC of fetal liver cells reaggregated with $CD45^{-}$ thymic stromal cells of either RAG-1-deficient or IL-7-deficient mice. TCR analysis was performed after 9 days for Vβ8, Vβ6, Vδ1, Vδ4, and p53.

on FCS/SSC pattern. However, since purified class II–positive stromal cells were able to support both V–DJβ and δ rearrangements (Figure 3B), our findings indicate that these thymic epithelial cells provide all components necessary for induction of this T cell commitment step. Consistent with this observation, no V–DJβ or V–DJδ rearrangements could be detected in fetal thymus from nude mice (data not shown), which lack class II–positive epithelial cells (Kingston et al., 1984).

Another feature that can be used to distinguish the different thymic stromal cell populations is differential cytokine expression (Moore et al., 1993). IL-7 production has been mapped to class II–positive thymic epithelial cells (Moore et al., 1993), and maintenance of the expression of this cytokine is not dependent on the presence of lymphoid cells (Moore et al., 1993). IL-7 is thus a potential feature that distinguishes class II–positive and class II–negative stromal cells. We therefore compared the ability of class II–positive epithelial cells from normal and IL-7-deficient mice (Von Freeden-Jeffry et al., 1995) to induce TCR-β and δ rearrangements in uncommitted fetal liver stem cells. The data shown in Figure 4 demonstrate that stromal cells from IL-7-deficient mice are entirely unable to induce TCR-β and δ gene rearrangements in fetal liver stem cells. This defect

is consistent with earlier reports that described IL-7 as a physiological survival factor for early lymphoid progentiors (Murray et al., 1989; Watson et al., 1989; Peschon et al., 1994; Moore and Zlotnik, 1995). Others (Muegge et al., 1993) have suggested that IL-7 is responsible for directly inducing TCR-β rearrangements; however, the data in that report are also consistent with an effect of IL-7 on selective outgrowth of precursors that have already rearranged TCR genes. In fact, the latter view is more in keeping with the results of others (Moore and Zlotnik, 1995). At least two studies, however, are consistent with the notion that IL-7 may have a direct effect on TCR-γ chain rearrangements (Appasamy et al., 1993; Maki et al., 1996). In the present study, IL-7 emerges as one of the unique features provided by class II–positive epithelial cells, instrumental in creating conditions for TCR-β and δ gene rearrangements to occur, presumably by supporting survival.

V–DJβ and δ Chain Rearrangements Can Be Induced in Purified CD117$^+$CD45$^+$ Fetal Liver Progenitors

The finding that TCR-β and δ chain rearrangements can be induced in fetal liver precursors by purified CD45-negative, class II–positive thymic epithelial cells (Figure 3B) raises the question of why both class II–negative mesenchymal cells and class II–positive thymic epithelial cells were found to be required for development of day 14 fetal thymocytes into mature αβ T cells (Anderson et al., 1993). Although it was not defined whether the target population in day 14 thymocytes represented committed or uncommitted precursors, these findings may at face value seem contradictory with our findings: day 14 fetal thymocytes contain already committed T cell precursors (Moore and Zlotnik, 1995; Zúniga-Pflücker et al., 1995) and complete V–DJ rearrangements (Figure 1A), and might thus be predicted to be subject to less stringent requirements than the fetal liver precursors used in our studies, in which no V–DJβ or δ rearrangements could be detected (Figure 1A). Alternatively, the intrathymic precursors used in those experiments (Anderson et al., 1993) may have lost the ability to respond to signals from cortical epithelial cells, or do not display that ability under the conditions used.

An obvious difference between our experiments and those of Anderson et al. is that the fetal liver cells we have used may contribute unknown components, not represented in the fetal thymocyte preparations used by Anderson et al. To address the possible contribution of any nonhematopoietic cells in the fetal liver input cells, fetal liver cells were fractionated by fluorescence-activated cell sorting (FACS) based on expression of CD117 and CD45 (Figure 5A) and subjected to reaggregate culture with total CD45-negative or purified CD45-negative, class II–positive fetal thymic stromal cells. Figure 5B shows that TCR-β and δ chain rearrangements were induced in both total fetal liver cells and fetal CD117$^+$CD45$^+$ hematopoietic precursors. Thus, CD117$^+$CD45$^+$ fetal liver cells contain all the necessary elements that, together with class II–positive thymic epithelial cells, allow T cell commitment. Besides induction of TCR gene rearrangement, we also evaluated further development along the T cell lineage from fetal liver stem cells. Figure 6 documents that both αβ$^+$ double positive and single positive T cells can be generated when CD117$^+$CD45$^+$ precursors are reaggregated with CD45$^-$class II$^+$ thymic stromal cells, comparable with the results with unsorted fetal liver and CD45$^-$ thymic stromal cells (see Figure 2). These data are consistent with the conclusion that all signals for T cell differentiation from uncommitted CD117$^+$CD45$^+$ fetal liver stem cells are contained within the subset of CD45$^-$class II$^+$, IL-7-producing fetal thymic stromal cells.

Discussion

The results presented here demonstrate that V–DJβ and V–DJδ rearrangements can be induced in fetal liver stem cells by interactions with a subset of CD45$^-$ thymic epithelial cells that distinguishes itself by expressing class II and producing IL-7. Thymic stromal cells that are CD45$^-$ but do not express class II or are deficient in IL-7 production cannot support this T cell commitment step. Future studies aimed at identification of the stromal cell–derived signals responsible for induction of TCR gene rearrangements can thus be focused on this unique subset.

Induction of TCR-β and TCR-δ rearrangements occurred with equal effectiveness by the stromal cell subsets we identified as being able to support rearrangements. Completion of commitment to either γδ or αβ T cell lineage development more likely occurs at a later stage, but how this is regulated is a matter of much debate (Dudley et al., 1994, 1995; Kang et al., 1995; Livak et al., 1995; Wilson et al., 1996). Expression of a TCR-β chain is an absolute determinant of T cell commitment (Mombaerts et al., 1992a; Kisielow and Von Boehmer, 1995), but not of αβ versus γδ T cell lineage diversification: V–DJβ rearrangements are frequently in-frame in γδ cells (Dudley et al., 1995). Compatible with a successive rearrangement model, V–JCγ rearrangements are frequently nonproductive in αβ cells (Kang et al., 1995), but the frequency of productive V–DJδ rearrangements in αβ T cells has been claimed to be either random (Wilson et al., 1996) or nonrandom (Dudley et al., 1995; Livak et al., 1995). Without question, however, a fair number of cells that undergo δ rearrangements will continue to become αβ cells (Dudley et al., 1994, 1995; Livak et al., 1995; Wilson et al., 1996). At what point the lineages then diverge, and whether regulation of the nature of the initial rearrangements by stromal cells plays any role, remains to be seen. The class II–positive thymic epithelial cells used in our experiments are of course heterogeneous, so different subsets may have supported TCR-δ versus TCR-β rearrangements. Differential support of lineage development does not, however, seem to be dictated by IL-7: neither TCR-β nor TCR-δ rearrangements were induced by stromal cells from IL-7-deficient mice (Figure 4). That stromal cell surface ligands might be involved in γδ versus αβ lineage diversification is illustrated by the recent observation that blockage of CD81, expressed by subcapsular cortical thymic stromal cells, results in inhibition of αβ but not γδ development in fetal thymic organ culture

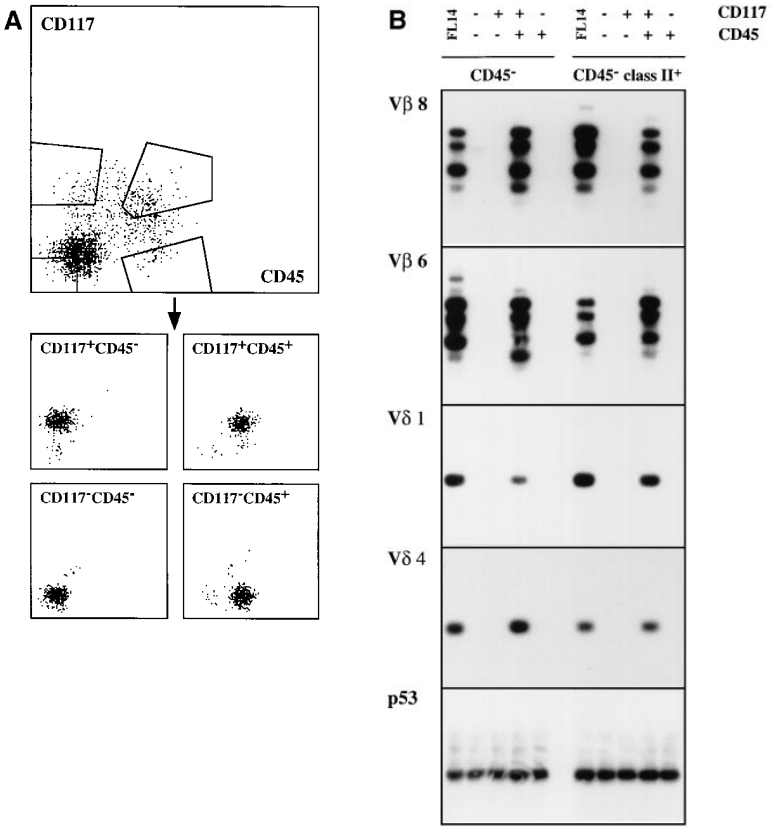


Figure 5. Class II–Positive Thymic Epithelial Cells Are Sufficient and Required to Induce TCR-β and δ Rearrangement in CD117$^+$ CD45$^+$ Fetal Liver Progenitors

Fetal liver was fractionated based on expression of CD117 and CD45. We reaggregated 2×10^5 total fetal liver cells or 2000 cells of any of the four subsets with either CD45$^-$ or CD45$^-$class II$^+$ thymic stromal cells.

(A) Flow cytometry of total fetal liver and subsets after sorting.

(B) PCR analysis was performed after 10 days for Vβ8, Vβ6, Vδ1, Vδ4, and p53.

(Boismenu et al., 1996). Since fetal thymi were used in these studies, the role of CD81 in the initiation of TCR gene rearrangements remains to be investigated.

The nature of the stromal cell support provided in the induction of rearrangements is likely to include both surface ligands and actively secreted cytokines. In support of a contribution of cytokines, we were unable to induce TCR gene rearrangements with metabolically inactive (Jenkins and Schwartz, 1987; Anderson et al., 1994a) ECDI-fixed thymic stromal cells (data not shown). Such ECDI-fixed cells, which require 3T3 cells to create coherent lobes (Anderson et al., 1994a), can still support positive selection (Anderson et al., 1994a). Class II–positive cortical epithelial cells were previously shown to express IL-7 and stem cell factor (Moore et al., 1993), and a role for these cytokines as physiological survival factors for early T cell precursors has been implicated (Peschon et al., 1994; Moore and Zlotnik, 1995; Rodewald et al., 1995; Von Freeden-Jeffry et al., 1995). Consistent with these observations, we now report that class II–positive epithelial cells from IL-7-deficient mice (Von Freeden-Jeffry et al., 1995) cannot support T cell commitment in fetal liver stem cells. In contrast, class II–negative thymic stromal cells, which do not produce IL-7 (Moore et al., 1993), fail to induce TCR gene rearrangement. Nevertheless, IL-7 alone cannot be the only contribution of CD45$^-$class II$^+$ fetal thymic stroma: we were also unable to induce rearrangements in reaggregates of class II–negative stromal cells supplemented with IL-7 (data not shown). Others (Zúniga-Pflücker et al., 1995) emphasized the requirements for IL-1 and tumor necrosis factor α in the differentiation of

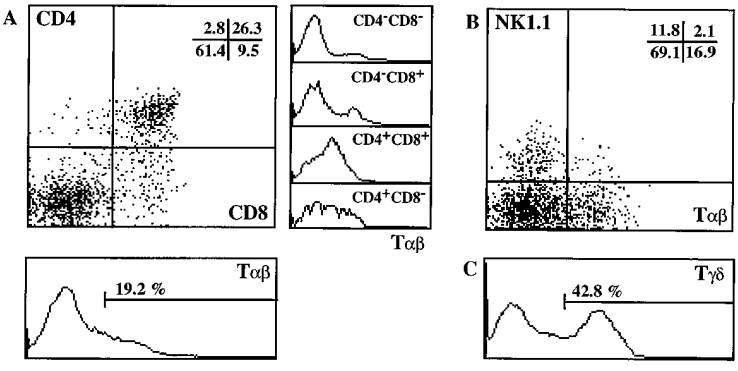


Figure 6. Class II–Positive Thymic Epithelial Cells Induce Complete T Cell Development in CD117$^+$CD45$^+$ Fetal Liver Progenitor Cells

Flow cytometry of a RTOC of class II$^+$ sorted thymic epithelial cells and CD117$^+$CD45$^+$ sorted fetal liver cells after 20 days in culture. Shown are CD4-PE, CD8-bio plus SA-Tri, and Tαβ-FITC (A); NK1.1-PE versus Tαβ-FITC (B); and Tγδ-FITC (C).

$CD25^-$ to $CD25^+$ thymocytes, and IL-1 is not produced by thymic epithelial cells (Moore et al., 1993). Our inability to induce differentiation with ECDI-fixed stroma would also be consistent with a role for cell surface ligands that, in contrast with peptide–MHC complexes (Jenkins and Schwartz, 1987; Anderson et al., 1994a), are ECDI sensitive. The observation that irradiated stromal cells are fully capable of induction of TCR-β or δ rearrangements (data not shown) would be in keeping with a role for such ECDI-sensitive ligands.

An additional hallmark of αβ T cell commitment might be expression of the pTα gene (Saint-Ruf et al., 1994; Fehling et al., 1995), which is present in committed T cell precursors in fetal thymus (Saint-Ruf et al., 1994; Bruno et al., 1995) and fetal blood (Rodewald et al., 1995), but not in either γδ cells or fetal liver (Bruno et al., 1995). Also, pTα-deficient mice develop γδ cells but not αβ cells (Fehling et al., 1995); an investigation of the role of thymic stroma in induction of pTα in fetal liver precursors would thus also be informative. Further identification of which cell surface ligands and cytokines from thymic stromal cells are responsible for the initiation of pTα expression and TCR gene rearrangements will eventually allow a definition of the thymic stroma–derived signals for these lineage commitment steps.

Experimental Procedures

Mice

Fetal C57BL/6 mice were used as a source of fetal liver stem cells. Fetal mice in which the *RAG-1* gene was disrupted (Mombaerts et al., 1992b) were used as a source of thymic stromal cells and bred under germ-free conditions. In some experiments, thymic stromal cells from IL-7-deficient mice were used (Von Freeden-Jeffry et al., 1995). To obtain timed pregnancies, female and male were mated overnight and the day of the plug was considered as day 0. Fetal liver from B6 mice was used at day 14 of gestation, and fetal thymus lobes from $RAG\text{-}1^{-/-}$ mice were dissected from embryos at day 15 of gestation.

MAbs

To remove cells of hematopoietic origin from thymic stromal cell suspensions, anti-CD45 (clone ER-MP33) (Leenen et al., 1990) in combination with anti–rat immunoglobulin-coated Dynal beads were used. Anti–MHC class II MAb (M5/114) (ATCC) was used to subdivide further CD45-negative thymic stromal cells by FACS. Further subsetting of class II–positive epithelial cells was performed with G8.8a (Farr et al., 1991), CDR1 (Rouse et al., 1988), NLDC145 (Kraal et al., 1986), and CD40 (Heath et al., 1994) MAbs, which were used as supernatant or purified protein in combination with sheep anti–rat immunoglobulin-FITC (Silenus). For flow cytometry analysis of T cell differentiation markers, directly conjugated MAbs from Pharmingen were used specific for TCR-αβ (clone H57-597), CD4 (clone RM4-5), CD8 (clone 53-6.7), NK1.1 (clone PK136), TCR-γδ (clone GL3), and several Vβ family members. Where appropriate, streptavidin–Tricolor (Caltag) was used as a second-step reagent. Enrichment of stem cells from fetal liver was performed in some experiments by sorting by FACStar Plus for $CD117^+CD45^+$ cells, using anti-CD117-FITC (clone 2B8) (Ikuta and Weissman, 1992) and anti-CD45-PE (clone 30F11.1), both also obtained from Pharmingen.

RTOC

Reaggregate thymic organ cultures (RTOCs) were based on an earlier description (Jenkinson et al., 1992), using the modifications outlined in Figure 1B. Fetal thymus lobes of RAG-1-deficient mice at day 15 of gestation were dissected and cultured in 1.35 mM dGuo on a filter and gelfoam to deplete cells of hematopoietic origin (Jenkinson et al., 1982). Dulbecco's medium was used and supplemented with nonessential amino acids, 10 mM HEPES, 4 mM L-glutamine, penicillin, and streptomycin (all from GIBCO), 5×10^{-5} M 2-β-mercaptoethanol, and 20% fetal calf serum. After 5 days in culture, dGuo was washed away and thymus lobes were trypsinized (0.25% trypsin in 0.02% EDTA; Sigma Chemical Co.) for 30 min at 37°C, followed by treatment with 10 μg/ml DNase (Sigma Chemical Co.) in complete medium for 10 min. Suspensions were made by vigorous pipetting and washed in complete medium, and residual cells of hematopoietic origin were depleted twice by magnetic beads (Dynal, GaRIg) coated with anti-CD45 MAb (MP33). In some experiments (see Results), further processing of thymic stromal cells involved sorting CD45-negative cells by FACStar Plus into MHC class II–positive and negative cells, using supernatant of M5/114 (ATCC) plus sheep anti–rat immunoglobulin-FITC, or treatment with the fixative ECDI (1-ethyl-3-[3′dimethyl-aminopropyl]carbodiimide), to render the cells metabolically inert (Anderson et al., 1994a).

The remaining stromal cell suspension was mixed with an equal number of fetal liver cells from B6 mice at days 13–14 of gestation (both 2×10^5 to 4×10^5 cells or 2000 $CD117^+CD45^+$ fetal liver cells) and spun down. After removal of the supernatant, the pellet was vortexed in a remaining volume of 2–4 μl and carefully dropped by using a crystal tip (Eppendorf) on a dry nucleopore filter resting on gelatine foam sponge and cultured as described for conventional FTOC (Jenkinson et al., 1992; Ramsdell, 1992). Reaggregated lobes were analyzed by PCR and flow cytometry at different timepoints as indicated in the figures.

PCR

Rearrangement of the TCR-β and TCR-δ loci was assayed at the genomic level as described (D'Adamio et al., 1992; Itohara et al., 1993). In brief, pools of reaggregate cultures were lysed in 50 μl of lysis mixture, containing 1× PCR buffer (67 mM Tris [pH 8.8], 6.7 mM $MgCl_2$, 10 mM 2-β-mercapto-ethanol, 6.7 mM EDTA, 16.6 mM $[NH_4]_2SO_4$), 0.45% NP-40, 0.45% Triton X-100, and proteinase K (100 μg/ml). Cells were incubated at 55°C for 1 hr, and subsequently proteinase K was inactivated at 96°C for 10 min. We used 5 μl of template in one PCR reaction of 50 μl total volume. Primers used to amplify rearranged DNA were as follows: Vβ8 (5′-TCCCTGAT GGGTAGAAGGCC-3′), Vβ6 (5′-GAA-GGCTATGATGCG-TCTCG-3′), and Jβ2.5 (5′-TAACACGAGGAGCCGAGTGC-3′); Vδ1 (5′-GGGA TC-CTGCCTCCTTCTACTG-3′), Vδ4 (5′-CCGCTTCTCTGTGAA-CT TCC-3′), and Jδ1 (5′-CAGTCA-CTTGGGTTCCTTGTCC-3′). PCR was performed as follows: denaturing, 1 min at 94°C; annealing, 1 min at 70°C for TCR-β or 1 min at 58°C for TCR-δ; extension, 1.5 min at 72°C; 33 cycles. PCR products were run on a 1.5% agarose gel, denatured in 0.4 N NaOH, blotted onto Hybond N+, and hybridized with a Jβ2-specific cDNA probe or a Jδ1-specific oligonucleotide (5′-GTTCCTTGTCCAAAGACGAGTT-3′) according to standard procedures.

p53 was amplified genomically as a template control using standard PCR conditions and hybridized with p53 cDNA. p53 forward primer, 5′-TCACTGCAT-GGACGATCTGTTGC-3′; p53 reverse primer, 5′-GATGATGGTAAGGATAGG-TCGGCG-3′.

Flow Cytometry Analysis

Reaggregated cultured lobes were put into 20 μl of PBA (1× PBS, 1% BSA, 0.02% NaN_3) in a 24-well plate and gently ground with the rubber plunge of a 1 ml syringe. Cells were resuspended and filtered over a mesh filter to remove cell clumps, aliquoted into wells of a round bottom 96-well plate (20,000–100,000 cells per well), and spun down (1200 rpm for 2 min). Cells were then resuspended in 20 μl of anti-FcγRII/III (clone 2.4G2; final amount 1 μg/ml) to reduce nonspecific staining, and incubated for 10 min at 4°C. Staining for specific antigen expression was then performed for 20–30 min at 4°C. Next, cells were washed in 100 μl of PBA and incubated if necessary with second-step reagent. Finally, all samples were washed twice and resuspended in 100 μl of PBA.

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